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Human cyclin F

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Cyclins are important regulators of cell cycle transitions through their ability to bind and activate cyclin-dependent protein kinases. In mammals several classes of cyclins exist which are thought to co-ordinate the timing of different events necessary for cell cycle progression. Here we describe the identification of a novel human cyclin, cyclin F, isolated as a suppressor of the G_1/S deficiency of a *Saccharomyces cerevisiae cdc4* mutant. Cyclin F is the largest cyclin, with a molecular weight of 87 kDa, and migrates as a 100–110 kDa protein. It contains an extensive PEST-rich C-terminus and a cyclin box region that is most closely related to cyclins A and B. Cyclin F mRNA is ubiquitiously expressed in human tissues. It fluctuates dramatically through the cell cycle, peaking in G_2 like cyclin A and decreasing prior to decline of cyclin B mRNA. Cyclin F protein accumulates in interphase and is destroyed at mitosis at a time distinct from cyclin B. Cyclin F shows regulated subcellular localization, being localized in the nucleus in most cells, with a significant percentage of cells displaying only perinuclear staining. Overexpression of cyclin F, or a mutant lacking the PEST region, in human cells resulted in a significant increase in the G_2 population, implicating cyclin F in the regulation of cell cycle transitions. The ubiquitous expression and phylogentic conservation of cyclin F suggests that it is likely to co-ordinate essential cell cycle events distinct from those regulated by other cyclins.

Key words: CDC4/cell cycle/CLB4/cyclin/cyclin-dependent kinase

Introduction

The cell cycle is composed of a series of complex and dynamic events whose temporal and spatial co-ordination is essential for proper duplication of cells. In eukaryotic cells, the cyclin-dependent kinases (Cdks) appear to be the primary regulators through which major cell cycle transitions are co-ordinated. Cdks are regulated by phosphorylation and association with activating and inhibitory subunits (reviewed in Hunter, 1993; Nasmyth and Hunt 1993; Pines, 1993; Sherr, 1993; Solomon, 1993). As the name implies, the activity of these kinases, and their subcellular localization and substrate specificity, depend upon the presence of a regulatory subunit called cyclins. Cyclins were first identified in synchronously fertilized sea urchin eggs (Evans *et al.*, 1983) as proteins whose amounts increased during interphase and then abruptly decrease at each mitotic division. That cyclins can promote particular cell cycle transitions was first demonstrated by the fact that injection of clam cyclin A mRNA could cause *Xenopus* oocytes arrested in prophase of meiosis I to enter mitosis and mature into eggs (Swenson *et al.*, 1986). It was subsequently found that cyclin B and Cdc2 (Cdk1) were the active agents of M phase promoting factor (MPF), a protein kinase purified using an oocyte maturation assay (Dunphy *et al.*, 1988; Lohka *et al.*, 1988; Draetta *et al.*, 1989; Labbe *et al.*, 1989).

Initially cyclins were defined on the basis of their periodicity of expression. Subsequently, they were found to contain a conserved amino acid sequence motif known as the cyclin box (Minshull *et al.*, 1989; Pines and Hunter, 1989; Westendorf *et al.*, 1989). All proteins now designated as cyclins contain this structural motif, which has been shown to contain information necessary for binding and activation of Cdks (Kobayashi *et al.*, 1992; Lees and Harlow, 1993; Zheng and Ruderman, 1993). Other motifs conserved in subsets of cyclins are sequences that confer ubiquitin-dependent degradation, termed 'destruction boxes' and PEST sequences. Destruction boxes are sequences found in cyclins A and B that allow for rapid proteolytic destruction at distinct points in the cell cycle (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Luca *et al.*, 1991; Lorca *et al.*, 1992). In the case of cyclins A and B, their destruction, as well as synthesis, is necessary for cell cycle progression. Expression of cyclins A or B lacking the destruction box arrests the cell cycle in mitosis (Murray *et al.*, 1989; Luca *et al.*, 1991; Gallant and Nigg, 1992). PEST sequences are proline-, glutamic acid-, serine- and threonine-rich regions found in many proteins and are thought to result in instability (Rogers *et al.*, 1986). Removal of PEST regions can increase the levels of cyclins (Tyers *et al.*, 1992; Hadwiger *et al.*, 1989). PEST regions are thought to allow protein levels to closely parallel mRNA abundance.

Cyclins fall into classes based upon their structural similarity, timing of expression and functional points within the cell cycle. Currently seven classes of cyclins have been reported in animal cells and these have been designated A–E, G and H; several of these classes have multiple members. Cyclins A and B are considered mitotic cyclins, being primarily expressed in G_2; however, they are functionally distinct. Cyclin A forms complexes with Cdc2 (Draetta *et al.*, 1989; Minshull *et al.*, 1990) and Cdk2 (Tsai *et al.*, 1991; Elledge *et al.*, 1992) and is required for both DNA replication and mitosis (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992). Cyclin B forms complexes exclusively with Cdc2 and is

required for entry into mitosis (reviewed in Norbury and Nurse, 1992). Cyclin A/Cdk complexes are localized in the nucleus and are active (Pines and Hunter, 1991; Cardoso *et al.*, 1993). In contrast, cyclin B/Cdk complexes first accumulate in the cytoplasm (Pines and Hunter, 1991) and are inactive due to inhibitory phosphorylation on threonine 14 and tyrosine 15 (reviewed in Solomon, 1993). At prophase, these complexes are activated and enter the nucleus rapidly just prior to nuclear envelope breakdown (Pines and Hunter, 1991; Gallant and Nigg, 1992; Bailly *et al.*, 1992; Ookata *et al.*, 1992). Additionally, cyclin A is synthesized and destroyed slightly earlier than B-type cyclins (Westendorf *et al.*, 1989; Pines and Hunter, 1990; Minshull *et al.*, 1990).

Cyclins D and E, considered G_1 cyclins, have been isolated by several methods (Lew *et al.*, 1991; Koff *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991), including their ability to complement G_1 cyclin deficiency in *Saccharomyces cerevisiae*. Cyclin D1 is required for passage through G_1 (Baldin *et al.*, 1993; Lukas *et al.*, 1994). Equivalent experiments have not been performed for cyclin E in mammals; however mutants in the *Drosophila* cyclin E homolog arrest cells prior to entry in S phase, indicating an essential role in the G_1-S phase transition (Knoblich *et al.*, 1994). Increased expression of cyclin D1 or cyclin E can independently shorten the G_1 phase of the cell cycle (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Jiang *et al.*, 1993; Resnitzky *et al.*, 1994), suggesting a rate limiting role in controlling passage through the restriction point. It is not clear whether they are shortening the same part of G_1. Despite these similarities, they are also functionally distinct. Cyclin D1 binds Cdk4 (reviewed in Sherr, 1993) and Cdk6 (Meyerson and Harlow, 1994), is growth factor inducible and its mRNA levels remain constant through the cell cycle in the presence of serum. Cyclin E associates with Cdk2 and shows cell cycle regulated transcription and abundance in G_1 (Koff *et al.*, 1992). Much less is known about the functions of cyclins C and G and their kinase partners are not known (Lew *et al.*, 1991; Leopold and O'Farrell, 1991; Lahue *et al.*, 1991; Tamura *et al.*, 1993).

Presumably by binding and activating Cdks, cyclins impart information to the cell which results in regulation of particular cell cycle transitions. There is a growing number of mammalian Cdks and Cdk-related molecules: the cyclin partners of many of these have not been identified (reviewed in Pines, 1993). It is therefore likely that many more cyclins remain to be discovered. The fact that a greater number of cyclins are currently known in *S.cerevisiae* also suggests that mammals are likely to contain as yet unidentified cyclins.

The cyclins identified in *S.cerevisiae* have been characterized genetically. A group broadly defined as G_1 cyclins includes *CLNs 1–3*, *HCS26* and *ORFD*, which are primarily expressed in or have genetic roles in G_1. The $S/G_2/M$ cyclins include *CLBs 1–6*. *CLB1* and *CLB2* are thought to be the counterparts of mammalian cyclin B because of their structural similarity and conserved timing of expression. Although a cyclin A structural counterpart has not been identified, *CLB5* and *CLB6* appear to play an important role in the timing and execution of S phase and may be functionally equivalent. *CLBs 1–4* have been shown to play roles in mitotic events (Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992). A deletion of all six *CLB* genes causes cells to arrest past START but prior to S phase; they contain unreplicated DNA and multiple buds (K.Nasmyth, personal communication). This phenotype is similar to that found in *cdc4* and *cdc34* mutants at their non-permissive temperatures (Hartwell *et al.*, 1973; Goebl *et al.*, 1988). In a genetic selection designed to identify human and yeast genes capable of suppressing *cdc4* mutants, we identified a novel human cyclin, cyclin F and a yeast cyclin, *CLB4*, suggesting a defect in cyclin function in *cdc4* mutants. In this report we focus on the characterization of this novel human cyclin. Cyclin F is most closely related to cyclin A with similarity stretching throughout and beyond the cyclin box. Cyclin F has many other properties in common with known cyclins such as a PEST region, cell cycle-regulated mRNA and protein abundance, regulated subcellular localization and the ability to alter cell cycle progression when overproduced. Based on these properties, cyclin F belongs to a new, evolutionarily conserved class of cyclins that is likely to perform a distinct role in regulating the human cell cycle.

Results

Isolation of human cDNAs that rescue the temperature sensitivity of cdc4

Many of the genes that control the cell cycle have been functionally conserved throughout eukaryotic evolution. A number of important human cell cycle genes, e.g. Cdc2Hs (Lee and Nurse, 1987), Cdk2 (Elledge and Spottswood, 1991) and cyclins C, D and E (Lew *et al.*, 1991; Koff *et al.*, 1991; Xiong *et al.*, 1991) have been identified by complementation of yeast *cdc* mutants. We sought to identify new components required for human cell cycle transitions using *S.cerevisiae* mutants that function after START but prior to initiation of DNA replication. *CDC4* encodes a protein with β-transducin repeats that is required for entry into S phase (Choi *et al.*, 1990; Yochem and Byers, 1987). Temperature-sensitive *cdc4* mutants initially arrest as large budded cells with a 1C DNA content, but continue to produce buds with a periodicity of one per cell cycle, resulting in a multiply budded cell arrested prior to S phase. We screened a human HepG2 cDNA library in a yeast expression vector, pAB23BXN (Schild *et al.*, 1990) for clones capable of rescuing the temperature sensitivity of a *S.cerevisiae cdc4-1* mutant. Selection performed on 3×10^6 library transformants produced 50 Ura^+ colonies that grew at 34°C (Figure 1A). Colonies were tested for plasmid dependency by streaking on media containing 5-fluoro-orotic acid, which selects for growth of cells that have lost *URA3* containing plasmids, and testing growth at 34°C. Thirteen that were plasmid dependent for growth at 34°C were recovered in *Escherichia coli* and found to carry related cDNA inserts by hybridization analysis.

Cyclin F—a novel class of human cyclins

The sequence of the longest cDNA (3130 bp) revealed a conceptual translation product of 786 amino acids with a predicted molecular weight of 87 kDa (Figure 2A). No in-frame stop codon was found upstream of the first methionine. 5′ rapid amplification of cDNA ends

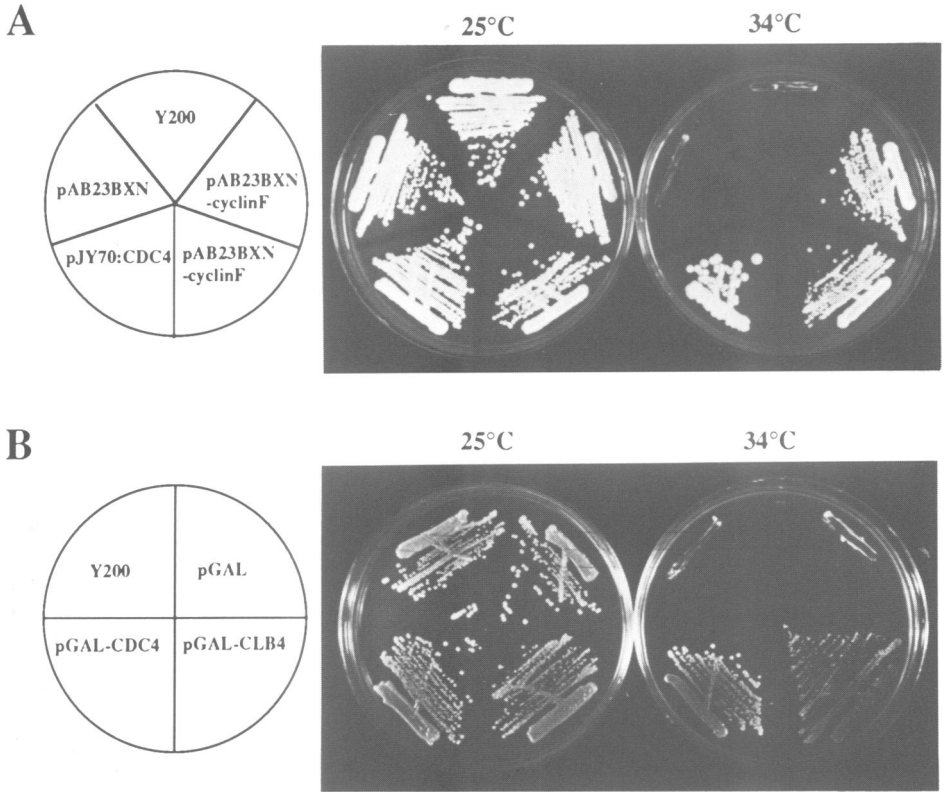

Fig. 1. (**A**) Rescue of the temperature lethality of *cdc4-1* cells by human cDNAs. Y200 (*cdc4-1 ura3-52*) cells with plasmids containing *CDC4* (pJY70), or expressing the human cDNAs (pAB23BXN−cyclinF),or vector alone (pAB23BXN), were struck on YPD medium, incubated at 25 or 34°C for 3 days and photographed. (**B**) Clb4 suppression of *cdc4-1* temperature sensitivity. Y200 cells with plasmid containing vector alone (pGAL1), pGAL-CDC4 or pGAL-CLB4 were struck on YP galactose media and incubated at 25 or 34°C for 4 days.

(5′RACE) was used to obtain further sequence in the putative 5′ untranslated region and identified a product only two nucleotides longer than our longest cDNA. The first methionine is within the Kozak consensus sequence (Kozak, 1986) and is likely to be the bona fide initiation codon for two reasons. First, human genomic clones for this cDNA were independently identified in a search for the polycystic kidney disease gene (A.M.Frischauf, S.Reeders and B.Kraus, personal communication) and sequences immediately upstream of our initiation codon were found to comprise a GC island indicative of promoter regions. Second, the same group has isolated the mouse cDNA and its sequence is reported to have an in-frame stop codon 5′ to the same initiating methionine.

A search of GenBank for amino acid sequences with homology to the conceptual translation product of our cDNA revealed a striking relatedness to the cyclin family of proteins. It also contains a highly PEST-rich sequence in the carboxy-terminal third of the protein, indicating that it is likely to be a metabolically unstable protein. This protein was designated as cyclin F. It is 40, 34, 30 and 27% identical to human cyclins A, B, D and E, respectively, in the cyclin box (Figure 2B), the primary region of sequence conservation among cyclins. Although it shares the strongest sequence conservation with cyclins A and B, it lacks the cannonical 'destruction box' sequences. Two putative nuclear localization sequences were identified: at the N-terminus and between the PEST sequence and the cyclin box (Figure 2A). Cyclin F encodes the largest cyclin (87 kDa) identified so far.

Overproduction of Clb4 can also suppress cdc4-1

Among yeast cyclins, none appear to be more closely related to cyclin F than to cyclins A and B. However, the suppression of *cdc4-1* by cyclin F suggested a possible genetic connection between a *S.cerevisiae* cyclin and the *CDC4* gene product. To search for a possible yeast cyclin F homolog, a *S.cerevisiae* cDNA expression library under *GAL* promoter control was introduced into Y200 (*cdc4-1*) cells and suppressors were selected at 34°C. Among non-*CDC4* encoding cDNAs were two classes of suppressor clones. One class encoded a known B-type cyclin, Clb4, previously identified as a high copy number suppressor of *cdc28-1N* (Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992; Figure 1B). Clb3 and Clb4 form a pair of highly related cyclins expressed predominately in S/G_2 that have been suggested to be involved in mitosis and spindle formation (Fitch *et al.*, 1992; reviewed in Nasmyth 1993). The second class of *cdc4* suppressors will be presented elsewhere.

Neither cyclin F nor Clb4 is capable of fully suppressing the temperature sensitivity of *cdc4-1* mutants. A significant proportion of *cdc4-1* cells carrying either cyclin F or *CLB4* expression plasmids still display the typical *cdc4* mutiple-budded phenotype at 34°C. They also have a much longer generation time than wild type cells: *cdc4*

Fig. 2. Sequence of cyclin F and alignment with other human cyclins. (**A**) DNA and predicted amino acid sequence of cyclin F cDNA. Nucleotide and amino acid numbers are given on the left. The initiating methionine (amino acid 1) and two putative nuclear-localization sequences are in bold face. Underlined is the most highly conserved region among cyclins known as the 'cyclin box'. The sequences underlined with a dashed line are the 'PEST'-rich regions. The stop codon is indicated by an asterisk. (**B**) Sequence comparison between cyclin F and human cyclins A (Wang *et al.*, 1990), B1 (Pines and Hunter, 1989), D1 (Lew *et al.*, 1991) and E (Koff *et al.*, 1991) within the conserved region. Dashes indicate identical amino acids and periods indicate gaps. In the cyclin box region (underlined), sequence identity of cyclin F is 40% with cyclin A, 34% with cyclin B, 30% with cyclin D and 27% with cyclin E. The similarity region between cyclin F and cyclins A and B extends well beyond the cyclin box. Over the entire 234 amino acid region shown, cyclin F shares 31% identity with A and 27% identity with B. The lower line indicates completely conserved residues among all five cyclins.

mutants expressing cyclin F at 34°C on glucose double every 3.5 h compared with 1.5 h for mutants harboring a *CDC4* plasmid. *cdc4* mutants overexpressing *CLB4* at 34°C on galactose double every 4.3 h compared with 1.7 h for wild type. Although this analysis failed to identify a yeast cyclin F structural homolog, the isolation of Clb4 as a *cdc4-1* suppressor suggests that cyclin F is capable of acting like a cyclin *in vivo*. Overproduction of human cyclin E, *CLN1* or *CLN2*, or the presence of the dominant *CLN3-2* mutation, failed to suppress *cdc4-1*, indicating that suppression of *cdc4* by cyclin F is not a general effect of cyclin overexpression (data not shown). However, it remains possible that the failure of other cyclins to suppress could result from differences in expression levels.

Cyclin F is also unable to suppress a *cdc4* deletion, further indicating that it is not completely bypassing *CDC4* function (see Materials and methods).

Cyclin F mRNA expression is tightly cell cycle regulated

One of the original defining characteristics of cyclins was their cell cycle related accumulation and destruction. Although this definition no longer holds for all cyclins, e.g. *CLN3* and cyclin D, when RNA expression does occur cyclically, the expression patterns correlate well with their temporal protein levels and their functional points within the cell cycle. Several cases in point are the correlation of the G_1 expression and function of cyclin E (Lew *et al.*, 1991; Koff *et al.*, 1992; Dulic *et al.*, 1992), the S/G2 phase expression and function of cyclin A (Pines and Hunter, 1990; Pagano *et al.*, 1992) and the G_2/M expression and mitotic function of cyclin B (Pines and Hunter, 1989). To gain insight into the function of cyclin F in the cell cycle we examined the expression of cyclin F mRNA using Northern analysis of synchronized cell population. Human peripheral blood lymphocytes (PBLs) in the G_0 phase of the cell cycle were stimulated to enter the cycle by phytohemagglutinin-P (PHA) treatment. Total RNA was extracted at various times after stimulation and analyzed by Northern blotting using CDC2 and cyclin F cDNAs as probes. Cyclin F message is almost undetectable in G_0 or G_1. It can first be detected 30 h post stimulation and reaches its maximal level of expression at 40 h (Figure 3A). CDC2, which has been previously shown to reach its highest expression at G_2/M (Elledge *et al.*, 1992), peaks several hours later than cyclin F. This indicates a peak of cyclin F expression in late S and G_2 phases of the cell cycle.

An alternative analysis of cyclin F expression was performed in samples of HEp-2 cells synchronized at the G_1/S boundary by a double thymidine block and harvested at timed intervals after release. Flow cytometric analysis was performed on propidium iodide stained cells at various times to determine DNA content as a control for synchrony. HEp-2 cells entered S phase immediately after release from thymidine, were predominately in G_2 at 8 h and completed mitosis at 10 h (Figure 3B). Northern blots were hybridized with cyclin F, cyclin A and cyclin B probes, as well as actin as a loading control (Figure 3C). The level of cyclin F mRNA is tightly cell cycle regulated. It begins to accumulate in S phase and sharply peaks in G_2, 6–8 h after release from the G_1/S block. Its accumulation coincides with initial expression of cyclin A and cyclin B, but cyclin F mRNA declines sharply while cyclin B mRNA is continuing to accumulate (Figure 3C–E). Levels of cyclin F mRNA varied >8-fold during the cell cycle (Figure 3E). The expression pattern of cyclin F coincides most closely with that of cyclin A, its closest relative by sequence, suggesting that it may function in S and G_2 like cyclin A.

The cell line and tissue specific expression of certain cyclins indicates that some of these are ubiquitous cell cycle regulators, like cyclins A and B, while others are likely to be involved only in certain cell types or developmental stages (Chapman and Wolgemuth, 1992; Won *et al.*, 1992). Northern blot analysis using poly(A)$^+$ RNA isolated from different human tissues indicates that cyclin F is expressed in all tissues examined, though some differences in expression levels are apparent (Figure 3F). These differences might reflect the fraction of cycling cells within a particular tissue. Cyclin F was also expressed in all seven cell lines tested (data not shown).

Identification of cyclin F protein

To begin a biochemical analysis of cyclin F, antibodies directed against a C-terminal synthetic peptide of cyclin F were generated. Production of antibodies against the full length protein was hampered by the difficulty of producing large amounts of cyclin F in bacteria. The specificity of the C-terminal antibodies was examined by immunoblot analysis using cyclin F prepared both by bacterial expression and by expression in COS-1 cells and by immunoprecipitation of *in vitro* translated ^{35}S-labeled cyclin F (Figure 4A and B). C-terminal antibodies, but not preimmune antisera, were able to detect cyclin F in these independent assays. Peptide competition blocked recognition of cyclin F in each assay (data not shown and see below). Although this antibody can detect approximately 1 ng of recombinant cyclin F by Western blot analysis, the levels in many cell lines fall just below our limits of detection, indicating low abundance or instability of cyclin F. However, by immunoprecipitation–Western analysis using large quantities of human cell lysate, we were able to detect cyclin F as multiple bands migrating at the size region of 100–110 kDa (Figure 4C). These bands are likely to represent authentic cyclin F because the same multiple banding pattern was also observed when recombinant cyclin F protein was expressed in COS-1 cells. The concordance in size between the endogenously expressed protein and recombinant protein further support the notion that the cDNA clone isolated contains the complete coding region. This size is greater than the predicted 87 kDa. However, most cyclins have an apparent molecular weight in SDS–PAGE larger than their predicted molecular weight. The fastest migrating species (100 kDa) co-migrates with *in vitro* translated cyclin F, suggesting that the slower migrating species might represent modified forms of cyclin F similar to those observed for other cyclins (Meijer *et al.*, 1989; Gautier and Maller, 1991; Gallant and Nigg, 1994).

Cyclin F protein levels fluctuate during the cell cycle

To determine whether cyclin F protein displays a cyclical pattern of abundance during the cell cycle, cyclin F protein levels were determined in synchronized cultures of HeLa cells. HeLa cells were chosen because the expression of cyclin A and B has been well characterized in these cells (Pines and Hunter, 1989, 1990; Dulic *et al.* 1992) and because cyclin F is sufficiently abundant to be detected directly by Western analysis in these cells. Immunoblotting of cell lysates from cultures synchronized using a double thymidine block revealed that the protein expression pattern mimics its message expression during the cell cycle. Cyclin F protein accumulates in interphase, reaches its maximal level at G_2/M phase and decreases around mitosis, beginning to accumulate again when cells enter S (Figure 5), a pattern very similar to that of cyclin A. Similar results were obtained using HEp-2 cells (data not shown). Although the timing of cyclin F disappearance is

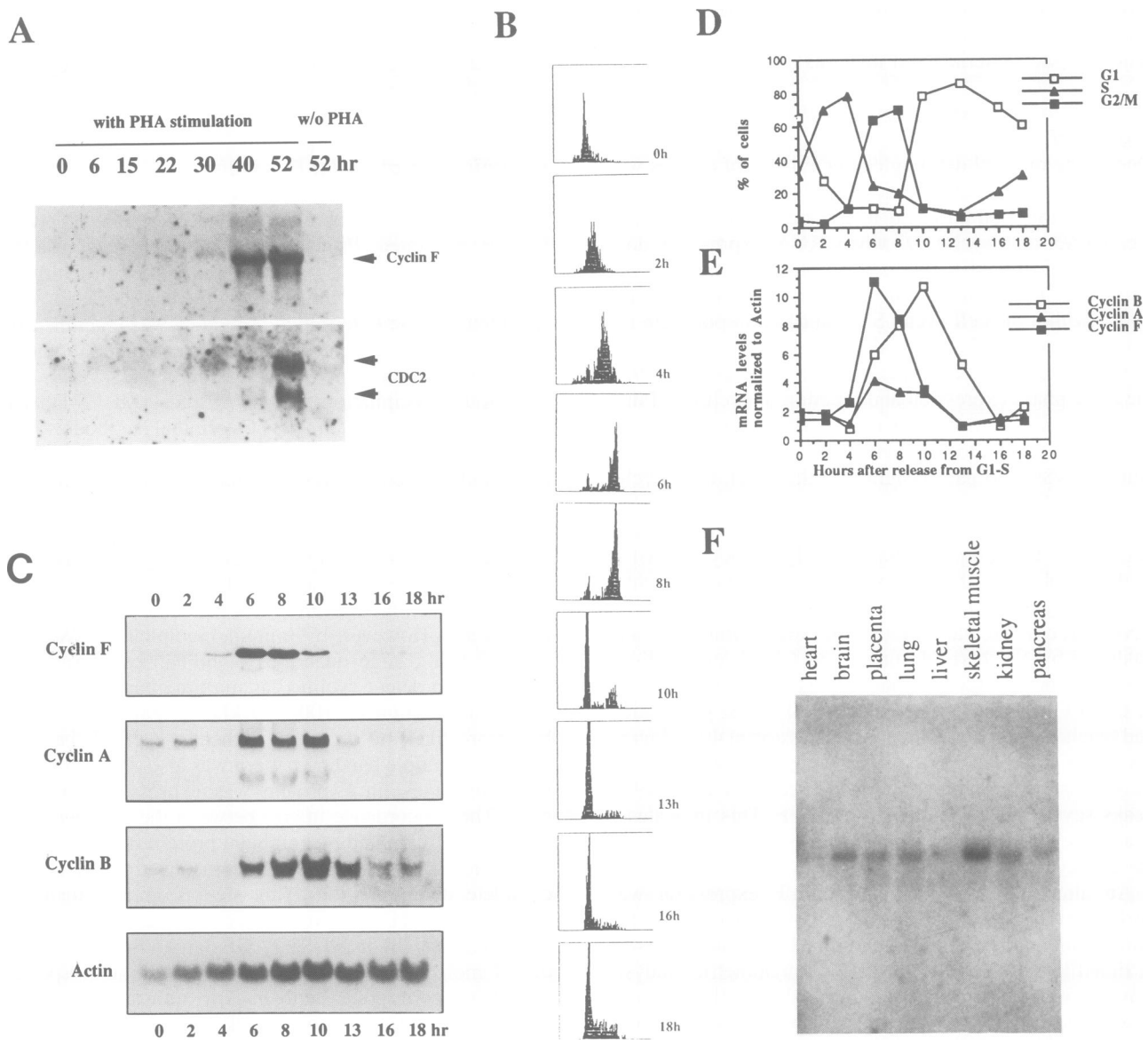

Fig. 3. Cyclin F mRNA expression patterns during the cell cycle and in human tissues. (**A**) Northern blot of cyclin F mRNA from human PBLs after stimulation with PHA. Total RNA was prepared at the indicated times after PHA stimulation and hybridized with a cyclin F probe and a CDC2 probe as indicated. (**B**) Flow cytometric analysis of HEp-2 cells at different times after release from a double thymidine block. The abscissa is the relative scale of DNA fluorescence and the ordinate shows relative cell number. (**C**) Northern blot of total RNA prepared from synchronized populations of HEp-2 cells described above sequentially hybridized with the indicated probes. Twenty micrograms of total RNA was loaded in each lane. (**D**) Percentage of cells in different stages during the cell synchrony experiment. (**E**) Relative amounts of cyclin F, cyclin A (2.2 kb message) and cyclin B mRNAs during the cell cycle normalized to actin mRNA. mRNA levels were quantitated using a betagen scanner. (**F**) Northern blot analysis of poly(A)$^+$ RNA from various human tissues. Two micrograms of poly(A)$^+$ RNA were loaded in each lane.

very similar to that for cyclin B, it is clearly distinct from cyclin B. In HeLa cells arrested with nocodazole (prometaphase arrest) after release from the double thymidine block, cyclin A and F levels were low while cyclin B levels were high (Figure 5).

Cyclin F shows regulated nuclear localization

Different cyclins display distinct patterns of subcellular localization. Cyclin A is predominately localized in the nucleus at replication foci (Pines and Hunter, 1991; Cardoso *et al.*, 1993). Cyclin B1 accumulates in the cytoplasm but is translocated into the nucleus just prior to nuclear envelope breakdown (Pines and Hunter, 1991; Gallant and Nigg, 1992; Bailly *et al.*, 1992; Ookata *et al.*, 1992; Pines and Hunter, 1994). Cyclin D is located in the nuclear region during the G_1 phase of the cell cycle but becomes immunologically undetectable when cells enter S phase (Lukas *et al.*, 1994). Due to the low abundance of cyclin F, we have been unable to directly visualize endogenous cyclin F protein unequivocally by immunofluorescence. However, using COS-1 cells transiently co-transfected with cyclin F and CD20 expression plasmids, we can readily detect cyclin F expression by indirect immunofluorescence. While a majority of the cells with cyclin F expression display nuclear localization (Figure 6A), we also observed a significant number of cells with perinuclear staining (Figure 6B) which varied from 10 to 30% in different experiments. The percentage of cells

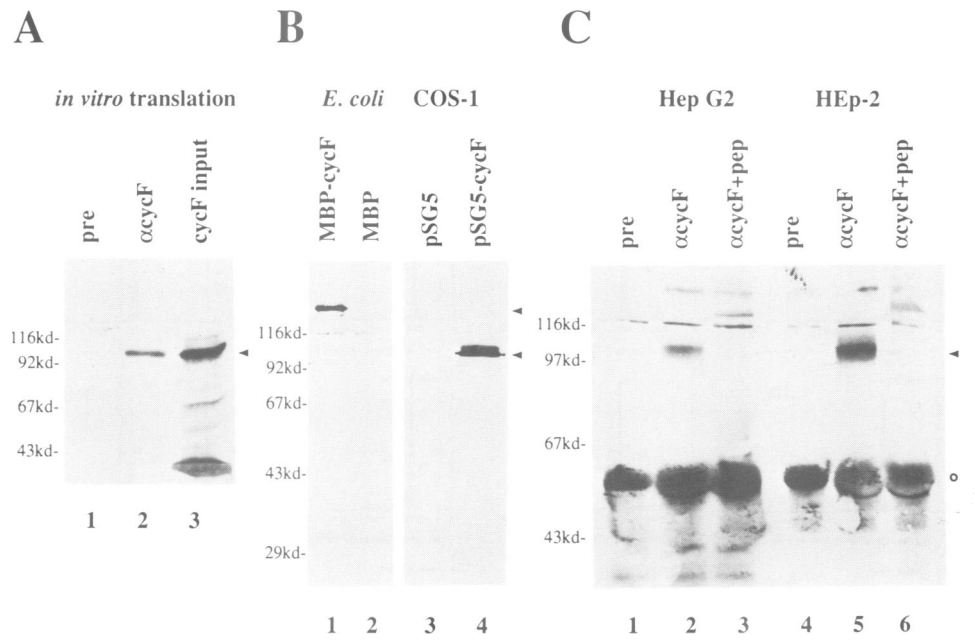

Fig. 4. Identification of cyclin F protein. (**A**) Immunoprecipitation of *in vitro* translated cyclin F. pre refers to preimmune sera, αcycF to cyclin F antisera and cycF input to the total amount of cyclin F *in vitro* translated protein used in each immunoprecipitation. (**B**) Western blot of recombinant cyclin F protein expressed in bacteria as a maltose binding protein (MBP)-cyclin F fusion or in COS-1 cells. pSG5 is a mammalian expression vector used to express cyclin F. (**C**) Detection of cyclin F protein in human cells. Cell lysates from HEp-2 or HepG2 were immunoprecipitated with preimmune sera (pre), cyclin F antibodies (αcycF), or cyclin F antibodies preincubated with the C-terminal peptide (pep) used for antibody production, fractionated on SDS−PAGE and immunoblotted with cyclin F antibodies. The circle indicates the position of IgG.

with perinuclear staining is unlikely to reflect the *in vivo* percentage because of the ability of cyclin F overexpression to perturb cell cycle distribution. The staining is specific for cyclin F since it is observed only in cells transfected with the cyclin F expression plasmid and it is specifically inhibited by preincubation of antibodies with antigen peptide (data not shown). This nuclear staining pattern is consistent with the putative nuclear localization signals found in cyclin F. However, the subset of cells displaying cytoplasmic staining in the perinuclear region indicates that cyclin F is likely to display cell cycle regulated translocation, as has been observed for cyclin B.

Cyclin F overexpression causes an increase in the G_2 cell population

Cyclin accumulation has been implicated in the control of timing of cell cycle transitions. In addition to the well documented effects of cyclin B on maturation of oocytes, overproduction of cyclins D1 and E has been shown to shorten G_1 (Ohtsubo and Roberts, 1993; Baldin *et al.*, 1993; Quelle *et al.*, 1993). Overexpression of cyclin A can overcome anchorage dependent growth (Guadagno *et al.*, 1993), stimulating passage into S phase during a normally extended G_1 period. To explore the *in vivo* role of cyclin F, we examined the effects of cyclin F overproduction on cell cycle transitions. Cyclin F under the control of the cytomegalovirus (CMV) promoter was transfected into C33A cells together with a CD20 expression plasmid. Analysis of the CD20-positive cells allows perturbations of the transfected population to be specifically measured within a larger population of cells not receiving plasmids (Heuval and Harlow, 1993). Cells were then stained with FITC-conjugated anti-CD20 antibodies and propidium iodide. Flow cytometric analysis of the DNA content of $CD20^+$ cells showed that the overproduction of cyclin F results in a significant and reproducible increase in the G_2/M cell population (Figure 7).

Several cell cycle transitions require not only the accumulation of cyclins, but also their subsequent degradation. For example, overexpression of cyclin B or expression of an undegradable cyclin B mutant causes cell cycle arrest at mitosis in *S.cerevisiae* (Ghiara *et al.* 1991; Surana *et al.* 1993) and in *Xenopus* egg extracts (Murray *et al.* 1989; Luca *et al.*, 1991). Further experiments suggest that the destruction of at least one protein other than cyclin B, perhaps another cyclin, is also required for the completion of mitosis (Holloway *et al.* 1993). Destruction of cyclin A appears to be necessary for activation of cyclin B proteolysis in mitosis (Luca *et al.*, 1991). Cyclin F does not contain an apparent destruction box in its N-terminal region; it is, however, highly PEST-rich in the C-terminal third of the protein. PEST-rich sequences are also found in most G_1 cyclins and several mitotic cyclins and are thought to promote general protein instability. The deletion of the PEST sequences in Cln3 resulted in the stabilization of Cln3 protein in the cells (Tyers *et al.* 1992). To examine whether removal of the PEST region could affect cyclin F function, we created a potentially stabilized cyclin F mutant lacking a large portion of the PEST region (Δ640–786) and measured its effect on cell cycle perturbation in the same transient assay. Transfection of cyclin F(Δ640–786) causes a significantly greater increase in the G_2/M population than does wild type cyclin F (Figure 7). These G_2/M increases are specific to cyclin F-transfected cells, because cells in the same sample which are not CD20 positive show proper cell cycle distribution (data not

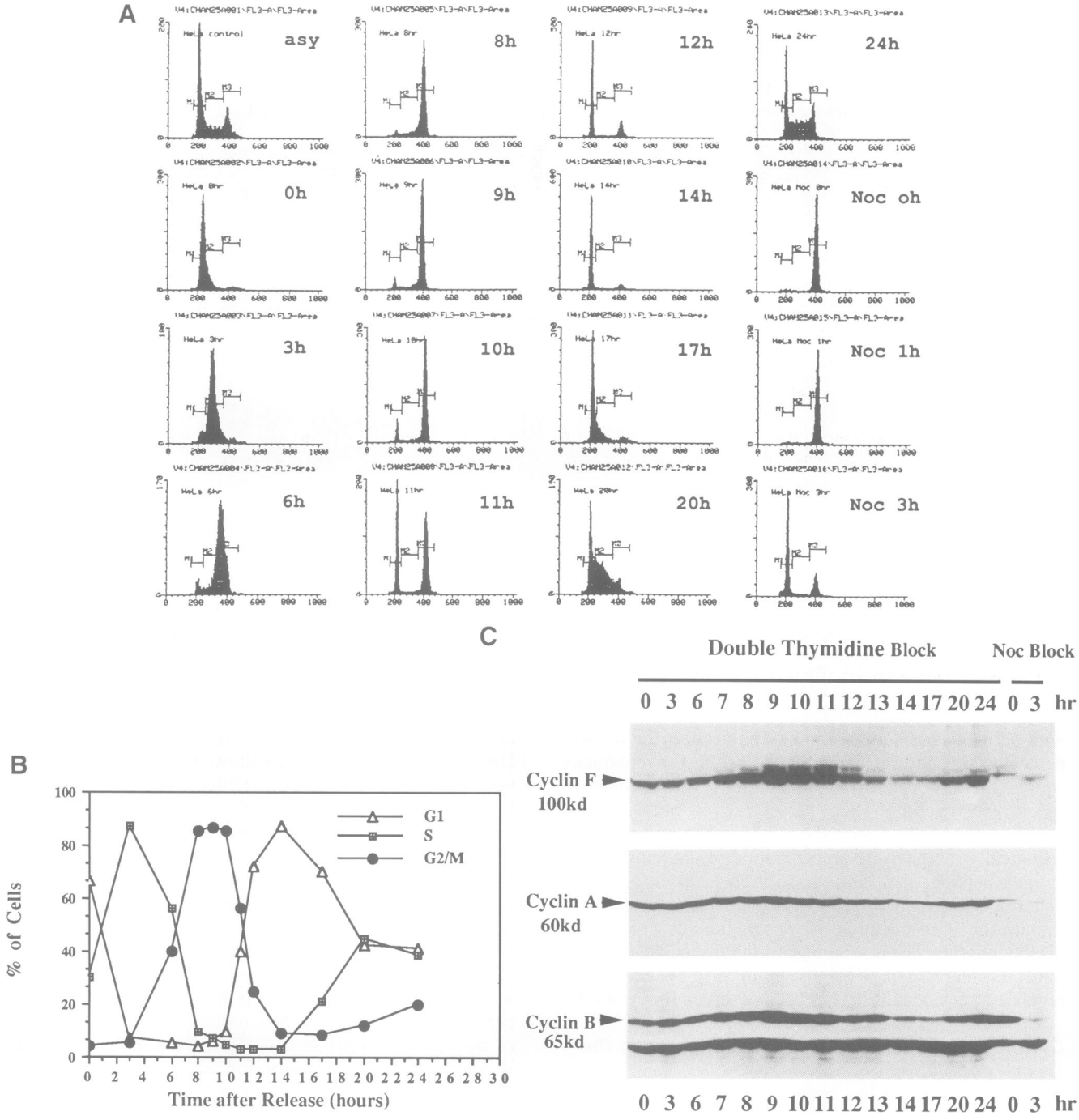

Fig. 5. Expression of cyclin F protein in HeLa cells synchronized using a double thymidine block alone or in combination with a subsequent nocodazole block. Cells in different stages of the cell cycle were obtained by taking samples at the indicated times after release from the block. (**A**) Flow cytometric analysis of the DNA content of cells at the indicated times (in hours) after release from the second thymidine block. Asy refers to a non-synchronized sample used as a control. The three panels labeled Noc refer to cells released from the double thymidine block into media containing nocodazole. The times refer to hours after release from the nocodazole block. (**B**) Quantitation of the percentage of cells in different stages of the cell cycle based on DNA content. (**C**) Immunoblot analysis of cell lysates prepared from the synchronized populations analyzed above. Equal loading was confirmed by ponceau S staining of the membrane after transfer and by a cross reacting protein observed with the cyclin **B** antibodies. Note that more time points were taken around G_2 and mitosis.

shown) while cells transfected with vector alone have a significantly lower proportion of cells in G_2/M. Furthermore, only minor differences are found between samples that were independently transfected with the same plasmid in the same experiment.

The phenotype of CD20 positive cells was further monitored by microscopic analysis of the state of chromosome condensation using propidium iodide staining. No increase in the proportion of cells with condensed chromosomes was observed for cells expressing high levels of cyclin F or the PEST-deleted form compared with vector alone, indicating that the increase in the population of cells with 4C DNA content was due to an increase in the G_2 and not the M population. This is distinct from that observed for cyclin B overexpression which causes mitotic arrest (Gallant and Nigg, 1992).

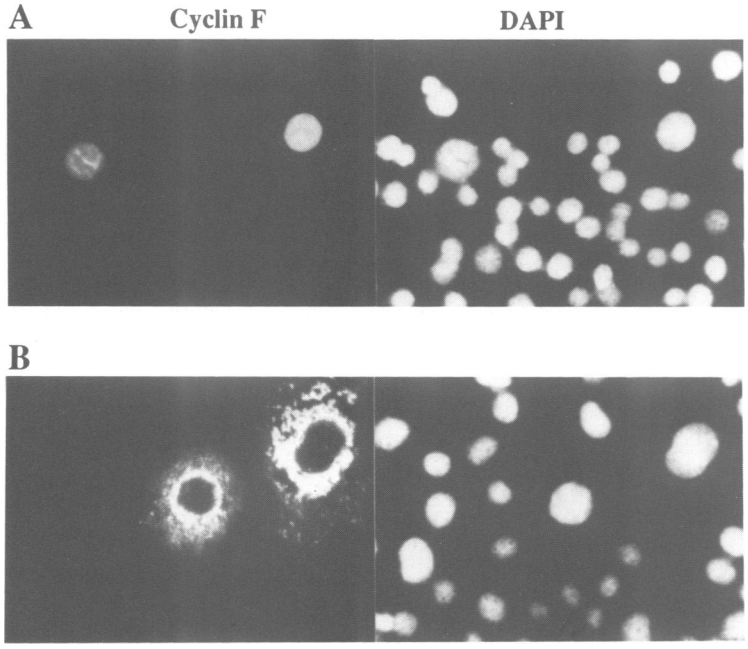

Fig. 6. Cyclin F displays regulated nuclear localization. COS-1 cells were transfected with pSG5-cyclin F and stained with anti-cyclin F antibodies 2 days later. Cyclin F was visualized by indirect immunofluorescence. DNA was visualized by staining with DAPI. (**A**) Cyclin F-expressing cells displaying nuclear localization of cyclin F. (**B**) An example of cyclin F-expressing cells displaying perinuclear localization.

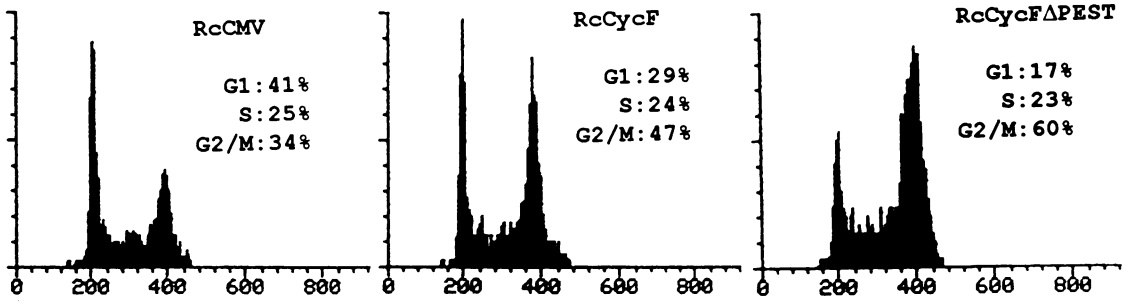

Fig. 7. Cyclin F overexpression or expression of PEST-deleted form of cyclin F in human cells results in an increase of cells with a G_2 DNA content. C33A cells were cotransfected with a CD20 cell surface marker expression plasmid, pCMVCD20, together with pRcCycF and pRcCycFΔPEST which direct the expression of cyclin F and cyclin FΔ640–786, repectively, or pRcCMV vector alone as a control. CD20 positive cells were analyzed for their DNA contents.

Discussion

We have identified a novel human cyclin, cyclin F, based on its ability to suppress a *cdc4-1* mutant when expressed in yeast. The assignment of cyclin F as a cyclin is based primarily upon its structural similarity to other known cyclins. It shows the highest sequence similarity to mitotic cyclins A and B. The similarity is found primarily in the cyclin box, although similarity between cyclin F and cyclins A and B also extends C-terminal to the cyclin box (Figure 2B). Another common cyclin feature conserved in cyclin F is the presence of an extensive PEST-rich region near the C-terminus. Like many cyclins, cyclin F has a strikingly periodic pattern of mRNA and protein expression within the cell cycle. Further support for assignment as a cyclin is its ability to act like a known cyclin, Clb4, when overexpressed in yeast. For these reasons, cyclin F is likely to function as a cyclin in mammalian cells.

Another hallmark feature of cyclins is their association with and regulation of cyclin-dependent kinases. The low abundance of cyclin F protein has hampered the detection of the catalytic partner of cyclin F, if such a partner exists. Attempts to observe cyclin F binding to Cdc2 and Cdk2 *in vitro* have failed (data not shown) and may indicate association with a different member of the growing family of Cdc2-related protein kinases (reviewed in Pines, 1993). Furthermore, we have been unable to detect protein kinase activity toward histone H1 and the retinoblastoma protein in cyclin F immunoprecipitates, although we can detect phosphorylation of cyclin F itself (data not shown). However, it is not clear whether this phosphorylation is due to a cyclin F-associated Cdk or other kinases present in the immunoprecipitates. Our inability to detect kinase activity on exogenous substrates may result from substrate specificity requirements, antibody interference in activity, inability to precipitate kinase bound forms or extremely low levels of active kinase.

Expression in PHA-stimulated T cells suggests that cyclin F is not involved in early cell cycle events, being first detected in S phase. HEp-2 and HeLa synchronization

experiments indicate that cyclin F is tightly cell cycle regulated, peaking in G_2. This timing and periodicity suggests that cyclin F may function in S and G_2 like cyclin A, which is involved in DNA replication and the G_2/M transition. Although reaching its maximal expression level at G_2 coincident with cyclin A, cyclin F mRNA decreases slightly earlier than cyclin A and much earlier than cyclin B. Cyclin F protein parallels its mRNA, accumulating in S and G_2 and being destroyed in mitosis coincident with cyclins A and B. Whether the disappearance of cyclin F is due to reduced transcription and extreme protein instability or whether it is due to a regulated destruction mechanism as observed for cyclins A and B remains to be determined. It is clear however, that if a destruction mechanism operates, it is distinct from that controlling cyclin B because cyclin F is destroyed at the nocodazole block while cyclin B is still abundant.

Cyclin F is predominantly nuclearly localized, consistent with the presence of putative nuclear localization signals. In addition, the presence of a distinct population of cells showing a perinuclear staining pattern indicates regulated translocation as observed for cyclin B1. We were unable to determine the precise cell cycle phase of the perinuclear stained cells and further experiments are required to determine the cell cycle timing of localization. However, because cyclin F overproduction causes accumulation of most cells in G_2, it is likely that the population with predominantly nuclear staining consists primarily of cells with a G_2 DNA content. It should be noted that the necessity of overproducing cyclin F for visualization could potentially lead to localization artifacts. However, many proteins, including cyclins, display proper localization when overexpressed (e.g. Jiang *et al.*, 1993; Gallant and Nigg, 1992; Pines and Hunter, 1994). The cell cycle regulation of expression and localization suggest that cyclin F is controlling the timing of an important cellular event.

Based on its timing of expression and sequence relationship to cyclins A and B, cyclin F is likely to play a role during S phase and G_2. This is further supported by the observation that overproduction of cyclin F results in an accumulation of cells in G_2. The interpretation of this genetic result is subject to a number of caveats, as is the case for all phenotypes resulting from overproduction. For example, G_2 accumulation could result from increasing the rate of passage through G_1 or S, as opposed to lengthening G_2. It is difficult to distinguish between these alternatives in a transient assay. Second, the phenotype could result not from increased cyclin F activity, but by a dominant interference in a different cyclin's ability to associate with a common kinase partner. Further experiments are required to establish the precise function of cyclin F in the cell cycle.

What implications for *CDC4* function result from the fact that cyclin F, Clb4 (and Clb2, R.Deshaies, personal communication) can suppress *cdc4* mutants? One possibility is that *cdc4* mutants are defective for expression of *CLB* cyclins necessary for DNA synthesis. This would be consistent with the similarity in phenotypes between mutants defective in all six *CLB* genes and *cdc4* mutants. Recently it has been demonstrated that *CLB* expression is required to turn off *CLN1* and *CLN2* transcription and activate its own expression (Amon *et al.*, 1993). Expression of mitotic cyclins could contribute to exit from START by both reducing *CLN* expression and increasing CLB expression, thus promoting DNA synthesis. *CDC34* encodes a ubiquitin conjugating enzyme involved in protein degradation and *cdc34* mutants have a terminal phenotype identical to *cdc4* mutants (Goebl *et al.*, 1988). *cdc34* mutants may be defective in destruction or inactivation of G_1 cyclins. If both G_1 cyclin destruction and transcriptional repression were required for exit from START, this might explain why *cdc4* and *cdc34* mutants have the same phenotypes. An alternative explanation for the suppression by cyclin F and *CLB4* is that an activating phosphorylation of Cdc4 by Cln/Cdc28 is defective in *cdc4-1* mutants and this phosphorylation can be restored to the mutant protein by mitotic cyclin/Cdc28 complexes.

Why was cyclin F isolated and not other human cyclins? One possibility is that other mitotic cyclins can suppress *cdc4*, but were missed because expression of cyclins A and B are toxic to yeast (S.J.Elledge, unpublished observation; Lew *et al.*, 1991). A second possibility is that cyclin F uniquely and specifically promotes a function in human cells that is defective in *cdc4* mutants and promoted by Clb4. *cdc4* mutants arrest past START and prior to S-phase with duplicated but unseparated spindle pole bodies. The *CDC4*-dependent separation of spindle pole bodies normally occurs just after START; however, the analogous events in mammals, separation of centrosomes, occurs at G_2/M. Clb3 and Clb4 have been proposed to be involved in the assembly of spindles (reviewed in Nasmyth, 1993). The suppression of *cdc4* by cyclin F and Clb4 could reflect their ability to restore the formation of functional spindles. The timing of cyclin F expression in mammalian cells is consistent with such a potential role.

Recently, the *Xenopus* homolog of cyclin F was isolated by cross-hybridization and shows extensive sequence conservation with the human protein (M.Howell and T.Hunt, personal communication). The regulated expression and localization of cyclin F together with its ubiquitous expression and phylogentic conservation suggests that it is likely to co-ordinate essential cell cycle events in all higher eukaryotic cells that are distinct from those regulated by cyclins A and B. More precise determination of its cellular roles will require the development of cells with conditional cyclin F function and awaits future experimentation.

Materials and methods

Media and yeast manipulations

Media were prepared as described in Rose *et al.* (1990). Transformation was performed as described previously in Elledge *et al.* (1991) using the lithium acetate method. Synthetic complete (SC) medium was used when plasmid selection was required. 5-FOA plates were made according to Boeke *et al.* (1984). To determine the ability of cyclin F to complement a *cdc4* null mutation, *HIS3* was cloned into pAB23-cyclin F and the resulting plasmid was introduced into Y201 [*cdc4::LEU2, his3-11,15* + pJY70(*CDC4 TRP1*)] selecting for histidine prototrophy. The resulting strain was grown in liquid under histidine selection and plated on SC−histidine media. Resulting colonies were replica plated onto SC-tryptophan plates to screen for colonies that had lost pJY70. No Trp^- colonies were found indicating that cyclin F could not suppress a *cdc4* null allele. This was consistent with the fact that cyclin F could suppress *cdc4-1* mutants for growth at 34°C but not at 37°C.

Libraries and vectors

The human cDNA library in pAB23BXN (2-μ, *GAP* promoter and *URA3*) was described in Schild *et al.* (1990). The *S.cerevisiae* cDNA

library in a *URA3 CEN* vector with a *GAL1* promoter was described in Liu *et al.* (1992). Cyclin F mammalian expression constructs were made in pRcCMV (Invitrogen) and pSG5 (Stratagene).

Cloning, sequencing and expression in bacteria

Nested deletions of cyclin F cDNA in Bluescript KSII (Stratagene) and sequencing were as described in Elledge and Davis (1987). The first two nucleotides of the cyclin F cDNA were obtained using a PCR based protocol called 5'RACE using a cyclin F specific reverse primer (CAUCAUCAUGTCGACAGACAGGCCTTCATTGTAGA) following a manufacturer's suggested protocol (GIBCO BRL). Multiple independent clones were isolated and sequenced.

For expression of cyclin F in bacteria, cyclin F full length cDNA was cloned into pMAL-c (New England Biolabs) to produce a maltose-binding protein (MBP)−cyclin F in-frame fusion. An exponentially growing culture of XL1-blue containing pMAL−cyclin F was harvested and lysed directly in 2× SDS Laemmli buffer. After boiling for 5 min, samples were run on SDS−PAGE.

Cell culture conditions, cell transfection and cell synchronization

Human PBLs were isolated from normal adult human blood by Ficoll−Hypaque gradient centrifugation and adherent depletion. Cells (1×10^6cells/ml) were maintained in DMEM with 10% fetal calf serum. Phytohemagglutinin (PHA) was added to a final concentration of 1 μg/ml and incubation was performed in a 5% CO_2 incubator.

COS-1 (SV40 transformed African Green monkey kidney cells), HEp-2 (human epidermoid carcinoma), HepG2 (human hepatoma) and C33A (human cervix carcinoma) cells were maintained in Dubecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum with 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml of penicillin in a 5% CO_2 incubator. Cells were transfected using the calcium phosphate precipitation method with 20 μg plasmid DNA for each 10 cm dish. Cells were treated with DNA precipitate for 18 h and analyzed 2 days after washing off the precipitates. For CD20-assisted cell sorting, 2 μg pCMVCD20 plasmid (Zhu *et al.*, 1993) were cotransfected with 20 μg pRcCMV-cyclin F or 20 μg pRcCMV vector alone for each 10 cm dish. Cells post-transfection were released from culture dish by incubation with 1% EDTA-Na for 5–10 min. After staining with FITC-conjugated anti-CD20 mAb (Becton Dickinson), cells were treated with RNase A and propidium iodide (PI) and analyzed as described in Heuvel and Harlow (1993). Approximately 2000 FITC-positive cells were analyzed for each sample.

HEp-2 cells were grown on 150 cm^2 flasks at 30% confluence and synchronized at the G_1/S boundary by a double thymidine block protocol (Rao and Johnson 1970) with first block for 16 h, a 10 h release and followed by a 14 h block. Cells were harvested at various times. 2×10^6 cells were fixed in ethanol and stained with PI for flow cytometry analysis to monitor the synchrony of cells. The remainder was used for isolation of total RNA. HeLa cells were synchronized using the same method but with 19, 9 and 17 h for 1st block, release and 2nd block respectively. Nocodazole (100 ng/ml) was used for arrest after releasing from the double thymidine block.

Northern analysis

Total RNA was extracted from cells using the RNAzol method (Biotecx Lab). RNA was resolved on an 1% formaldehyde−agarose gel, transferred onto a nylon membrane (Schleicher and Schuell) and probed with hexamer-labeled cyclin F, a 0.7 kb *Bst*X1 fragment from 1235–2061 bp, and full length cyclin A and cyclin B cDNA (Pines and Hunter, 1990). The human tissue blot (Clontech, Palo Alto, CA) contained 2 μg per lane of poly(A)$^+$ RNA isolated from indicated tissues.

Antibody production, purification and Western blot analysis

Antibodies against cyclin F were prepared by immunizing rabbits with a peptide corresponding to the C-terminus of cyclin F (EDMNLGLVRL) coupled to KLH via an appended N-terminal cystein. Affinity purified antibodies were generated by releasing antibodies from a cyclin F C-terminal peptide affi-10 gel column with 4 M guandine hydrochloride followed by dialysis against water and concentration by centriprep concentrator (Amicon). Cyclin F antibodies were used at 1:1000 or 1:10 000 dilutions with ProtoBlot AP system (Promega) for the immunoblot analysis shown in Figure 4 or the ECL system (Amersham) for the analysis shown in Figure 5. The production of cyclin A antibodies was described previously (Elledge *et al.* 1992). Cyclin B1 antibodies were obtained from Santa Cruz Biotech.

In vitro transcription, translation and immunoprecipitation

The TNT^{TM}-coupled reticulocyte lysate system (Promega) was used for *in vitro* transcription and translation of cyclin F cloned in pBluescript or pET 3d. pET3d−cyclin F was made by cloning PCR amplified cyclin F coding sequence (primers: GAACCCATGGGGAGCGGCGGCGTGGT and GCAAGGATCCACTGCGGCAAATGTGCTG) into *Nco*I−*Bam*HI-digested pET3d (Studier *et al.*, 1990). [^{35}S]methionine was purchased from ICN (Trans^{TM} label). For test immunoprecipitations, 3 μl of *in vitro* translation product were used in 400 μl NP-40 buffer (0.5% NP-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM PMSF, 20 mM EDTA, 25 μg/ml leupeptin) immunoprecipitated by 1 μl antiserum or 0.1 μg affinity purified antibodies. After a 2 h incubation on ice, protein A−Sepharose conjugate (Pharmacia) was added to collect antibody. Immunoprecipitates were washed three times with NP-40 buffer, boiled in 2× SDS sample buffer and resolved on SDS−PAGE. For each immunoprecipitation from human cells, six 80% confluent 10 cm dishes of HepG2 or HEp-2 cells were lysed in SDS−lysis buffer (0.2% SDS, 2% NP-40, 0.5% Na deoxycholate, 25 mM Tris pH 7.4, 50 mM NaCl and a cocktail of protease inhibitors and phosphatase inhibitors). Cell lysates were cleared by centrifugation for 20 min at 12 000 r.p.m. at 4°C. After a 2 h incubation with 5 μl antiserum, 50 μl protein A beads were added and rocked for 1 h at 4°C. Immunoprecipitates were washed once in lysis buffer and three times with washing buffer (25 mM Tris pH 7.4, 50 mM NaCl, 0.5% Na deoxycholate, 0.2% NP-40 and a cocktail of protease and phosphatase inhibitors) before analysis on SDS−PAGE.

Indirect immunofluorescence for subcellular localization

Cells 24 h post-transfection were trypsinized, plated onto a 2-well slide chamber (Nunc, Naperville, IL) and incubated for 24 h. Cells were fixed either with methanol for 2 min or with 3% formaldehyde for 5 min followed by permeablization in 0.5% Triton X-100 for 5 min. After preincubation of cells with 3% BSA in PBS for 2 h, cyclin F antibodies (1:1000) were added. After a 2 h incubation, cells were washed three times with 3% BSA in PBS. Texas red-conjugated donkey anti-rabbit IgG (1:250 dilution; Jackson ImmunoResearch Laboratories, Inc.) was used as secondary antibody and incubated for 1 h. Slides were washed three times with PBS, stained with 1 μg/ml DAPI for 3 min, washed four times with PBS and briefly air dried. For CD20 counterstaining, cells were incubated with FITC-conjugated anti-CD20 mAb for 15 min before fixation.

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The EMBL/GenBank accession number for cyclin F is U17105.